Research Article

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Efficient mAb production in CHO cells incorporating PEI-mediated transfection, mild hypothermia and the co-expression of XBP-1

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Abstract

BACKGROUND: Transient gene expression (TGE) provides a rapid way to generate recombinant protein biologics for pre-clinical assessment. Human embryonic kidney (HEK293) cells have traditionally been used for TGE; however, there is demand from industry for efficient, high-producing TGE systems that utilize Chinese hamster ovary (CHO) cells. A polyethyleneimine (PEI) -based TGE process has been developed for CHO cells using an episomal expression system to generate enhanced recombinant protein titers.

RESULTS: A five-fold improvement in monoclonal antibody (mAb) volumetric productivity was achieved by examining key parameters including transfection medium, cell density, transfection reagent, DNA : reagent ratio, the time of transfer to mild hypothermia and feeding strategy post-transfection. The *Epi*-CHO system allowed for a six-fold expansion in culture volume post-transfection without significantly affecting specific productivity. This system generates 400% more mAb per μg of plasmid DNA when compared with a non-episomal system. In addition, the use of X-box binding protein 1 to enhance secretion capacity and provide further improvements in mAb production with TGE was investigated.

CONCLUSION: Through optimization of key parameters, our results demonstrate the development of a low-cost, high-yielding, episomal TGE system that may be adopted during pre-clinical biologic drug development.

Keywords: CHO; mild hypothermia; monoclonal antibody; polyethylenimine; transient gene expression; XBP-1

INTRODUCTION

Cultured mammalian cells continue to be the dominant expression system for the production of recombinant protein-based pharmaceuticals.[1,2] This is mainly due to their capacity to efficiently produce biologically active, complex proteins such as monoclonal antibodies (mAbs). For clinical manufacturing these proteins are produced using high-yielding stable cell lines, which is an expensive and time-consuming process, typically taking 6–12 months. To rapidly screen large numbers of drug candidates during pre-clinical assessment, sufficient yields can be produced efficiently using transient gene expression (TGE) systems. TGE systems allow for the production of milligram to multi-gram quantities of recombinant protein in days rather than the many months taken to develop stable cell lines.[3,4] For widespread industrial acceptance, robust TGE processes must be developed that use animal component free reagents, offer high yields and are cost effective. In this work the development of an efficient system for the transient expression of recombinant mAbs in CHO cells is outlined.

The first critical parameter to address for optimized TGE is the efficient delivery of plasmid DNA into cells to facilitate higher yields of recombinant protein. Chemical-based transfection methods are routinely used rather than viral transduction and physical methods such as electroporation, due to their ease of use with suspension cells and their ability to be easily scaled to large-volume cultures. Common chemical delivery agents utilized for TGE include calcium phosphate,[5–10] cationic lipids[11–13] and cationic polymers. The cationic polymer polyethylenimine (PEI) is routinely used as a transfection reagent primarily because of its efficiency, cost-effectiveness and ease of use in large-scale transfections.[3,14,15] PEI functions as a transfection agent by efficiently condensing bound DNA resulting in the formation of polyplexes with a net positive charge, which are taken up by the mammalian cell through interactions with negatively charged cell surface molecules such as glycoproteins and proteoglycans expressed on the cell surface.[12,16–18] Polyplexes are transported into the cytoplasm by endocytosis and released following endolysosome rupture, which is induced by a 'proton sponge' effect.[15,19,20] The transport of polyplexes into the nucleus is believed to be an inefficient process and an area requiring further characterization due to its impact on transfection efficiency and recombinant protein expression.[14,21–24] PEI-mediated transfection represents

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an efficient and cost-effective method for large-scale TGE, however, careful optimization of critical transfection parameters is required to ensure that the maximum amount of recombinant protein is produced.[25–27]

In addition to efficient plasmid delivery, the TGE process should utilize defined, animal component free growth medium and supplements to mimic the conditions used during late-stage manufacturing in industry, with either batch or fed-batch conditions. These media formulations allow cells to attain high cell densities with elevated viability, making them ideal for prolonged TGE to facilitate increased protein production.[27,28] In this work an efficient PEI-mediated transfection process is combined with chemically defined (CD) media and feeds to increase recombinant protein titers.

Changes in the culture environment are also being integrated into TGE processes to improve recombinant protein production. Mild hypothermic (27–34 °C) culture conditions have been shown to facilitate higher titers with CHO cell-based stable and transient gene expression processes.[29–31] While not completely elucidated, these improvements most likely result from alterations in miRNA expression, enhanced mRNA stability and elevated culture viabilities through slower cell metabolism and a reduced rate of toxic metabolite accumulation (particularly lactate and ammonium).[31–35] More importantly, the effects of mild hypothermia appear to be temperature, cell line and protein dependent with recent reports demonstrating that detrimental effects on global protein synthetic capacity and cap-dependent mRNA translation in transfected CHO cells was only observed at 27 °C and not at 32 °C.[34,36,37]

The dilution of plasmid DNA into daughter cells after consecutive rounds of cell division post-transfection is a major factor limiting yields with TGE. The development of episomal-based expression systems has attempted to address this issue, as plasmid DNA is amplified and/or maintained extrachromosomally post-transfection using viral genetic elements. Many TGE studies have utilized modified HEK293 cells stably expressing Epstein-Barr virus (EBV) nuclear antigen 1 (EBNA-1) (HEK.EBNA), where EBNA-1 drives the replication and maintenance of transfected plasmid DNA containing the latent origin of replication, *OriP*.[38–40] While these elements do not provide an indefinite replicative effect, they are able to significantly prolong the period of gene expression.[25,26] To facilitate improvements in TGE with CHO cells (which are typically lower than those obtained with HEK293 cells), the *Epi*-CHO episomal-based transient expression system was developed.[41] This system utilizes a stable CHO cell line expressing the mouse polyomavirus (PyV) large-T antigen and plasmids containing the PyV origin of replication (*PyOri*) to promote the replication of plasmid DNA in CHO cells after transfection. In addition, it also employs EBNA-1 and *OriP* from EBV to facilitate the retention and segregation of plasmid DNA during cell division. Previous studies using the *Epi*-CHO system have demonstrated significant improvements in recombinant protein titers over non-episomal systems using lipofection in protein containing media.[41,42]

Finally, the possibility of improving cellular secretion capacity with TGE systems may provide an alternative approach to improving recombinant protein titers. The transcription factor X-box binding protein 1 (XBP-1) has been described as a key regulator of secretion[43,44] and the unfolded protein response (UPR) in mammalian cells.[45] During the UPR, the active isoform, XBP-1S (translated from the spliced mRNA of XBP-1), activates the expression of a series of genes involved in the secretory pathway, notably those that increase the content of the ER, golgi and mitochondria which in turn enhance total protein synthesis.[46,47] This makes XBP-1 an attractive engineering target to boost recombinant protein titers that may result from secretion bottlenecks.[48–51]

To increase recombinant protein production with CHO cell-based TGE, we describe in this report an optimized strategy for high-level mAb expression with the *Epi*-CHO system using PEI and CD media. This was achieved through the examination of critical transfection variables including the transfection reagent, cell density, DNA : reagent ratio and timing for transfer to mild hypothermic conditions post-transfection. Using these methods in combination with the *Epi*-CHO system we achieved a five-fold increase in recombinant mAb titer in a 15 day process compared with our initial conditions. In addition, it was demonstrated that through the co-expression of XBP-1, improvements in transient recombinant mAb titers can be achieved in combination with mild hypothermia.

METHODS

Plasmids

pEGFP-C1 (Clontech, Palo Alto, CA, USA), pBasic and pPyEBV have been described previously.[41] pPyEBV and pBasic were modified to express the heavy and light chain cDNA of the mAb Ab2 (an IgG1) to create pBasic-Ab2HC/pBasic-Ab2LC and pPyEBV-Ab2HC/Ab2LC, respectively. The cDNA for the spliced variant of murine XBP-1 was PCR-amplified from plasmid DNA template (forward primer 5′-GCTAGCATGGTGGTGGTGGCAGC-3′, reverse primer 5′-CTCGAGTTAGACACTAATCAGCTGGG-3′). The sequence was cloned into pCR2.1TOPO (Invitrogen), and then sub-cloned into the multiple cloning site of pcDNA3.1 (+) (Invitrogen) to create pcDNA3.1-XBP1.

Plasmid DNA for transfection was purified using either the PureLink HiPure Plasmid MaxiPrep Kit (Invitrogen, Carlsbad, USA) or PureYield™ Plasmid Maxiprep System (Promega, Madison, USA). Unless otherwise stated, DNA for transfections expressing Ab2 were composed of pPyEBV-Ab2HC : pPyEBV-Ab2LC : pEGFP-C1 at a ratio of 47.5 : 47.5 : 5 (w : w). Work performed by our group has demonstrated that the co-transfection of 2.5–5% pEGFP-C1 does not have any detrimental effects on Ab2 yields when compared with transfectants co-transfected with no pEGFP-C1 (data not shown).

Cell culture

Suspension CHO-T[41] and CHO-K1 (ATCC CCL-61) cells were maintained in CHO-S-SFMII (Invitrogen) serum-free (SF) medium or CD CHO CD medium (Invitrogen) supplemented to a final concentration of 8 mmol L^{-1} GlutaMAX (Invitrogen) and 0.4% (v/v) anti-clumping agent (ACA) (Invitrogen). CHO-T cells were also sequentially adapted to growth in SFM4CHO and CDM4CHO (HyClone, Logan, UT, USA), ProCHO5 and PowerCHO2 (Lonza, Walkersville, MD, USA) and EX-CELL 302 and EX-CELL CD CHO-3 (SAFC, Lenexa, KA, USA). Unless otherwise stated, cells were maintained at 37 °C in 7.5% CO_2 and 80% humidity. Cell density, viability and average cell diameter were determined using the Cedex HiRes Automated Cell Counter (Roche Innovatis, Bielefeld, Germany). Glucose, lactate and ammonium concentrations and pH in cultures were quantified using the BioProfile Flex (Nova Biomedical, Boston, USA). Note that due to the use of the modified glutamine variant GlutaMAX, actual glutamine concentrations could not be quantified accurately and are therefore not reported.

Cationic polymer-mediated transfection

On the day of transfection, cells in mid-logarithmic growth phase were diluted to 1.5×10^6 cells mL^{-1} in growth media (without ACA) (unless otherwise stated). Polyethyleneimine 'MAX' (referred to as PEI in this report) (Polysciences Inc, Warrington, PA) (prepared at 1 mg mL^{-1} in water) was complexed with plasmid DNA at a ratio of 3.5 : 1 (w : w) using 2 µg DNA mL^{-1} culture. Plasmid DNA and PEI were diluted in separate aliquots of OptiPRO SFM media (Invitrogen) (12.5% of initial culture volume) and incubated for 1 min at room temperature (RT). Both aliquots were then mixed together, incubated for a further 15 min at RT and then added to cells. This protocol was linearly scaled for all transfection volumes. All transfectants were incubated at 37 °C for 4–6 h post-transfection on an orbital shaker at 170 rpm. Cultures were then diluted with an equal volume of growth medium supplemented to achieve final concentrations of 0.4% (v/v) ACA, 100 µg L^{-1} IGF-1 (Novozymes, Adelaide, Australia) and 8 mmol L^{-1} GlutaMAX, with mAb expressing cultures then incubated at 32 °C unless otherwise stated.

Cationic lipid-mediated transfections

On the day of transfection, cells in mid-logarithmic growth phase were diluted to 1.5×10^6 cells mL^{-1} in growth media (without ACA). Lipofectamine 2000 and Freestyle MAX (Invitrogen) were complexed with plasmid DNA at a ratio of 2.5 : 1 and 1 : 1 (v : w), respectively, using 2 µg DNA mL^{-1} culture. Each component was diluted in separate aliquots of CHO-S-SFMII (Lipofectamine 2000) or OptiPRO SFM (Freestyle MAX) (12.5% of initial culture volume). With the former, components were incubated for 10 min at room temperature (RT), mixed together prior to a further 25–30 min incubation at RT and added to cells. For Freestyle MAX transfections, components were incubated for 1 min at RT, mixed together prior to additional 10 min incubation at RT and added to cells.

EGFP and mAb quantification

EGFP expression was quantified by flow cytometry using the FACSAria II (BD, San Jose, CA). In this report, transfection efficiency is defined as the percentage of viable cells expressing EGFP at 48 or 72 h post-transfection. mAb concentrations in culture supernatants were quantified using sandwich ELISA with Nunc 96-well MaxiSorp plates (Thermo Fisher Scientific, Roskilde, Denmark) as described previously.[52] The average cell-specific productivity over the duration of an expression study was calculated from the slope of the line of best fit for accumulated mAb (mg) plotted against the accumulated integral viable cell (IVC) number,

$$\text{Accumulated IVC} = \int X_v \, dt = \frac{(X_i + X_f) \times T}{2}$$

where X_v is the total number of viable cells (10^6 cells); X_f and X_i are the current and previous total number of viable cells, respectively; T is the time interval between the current and previous sample (days).

RESULTS AND DISCUSSION

Comparison of growth media and transfection reagent for successful TGE

The use of advanced CD and SF media provides conditions for prolonged cell growth and viability post-transfection, as well as generating higher titers of recombinant proteins.[27,53] The ability of PEI to transfect CHO-T cells adapted to a range of commercially available CD and SF media was analyzed. Cells were transfected with pEGFP-C1 in six-well plates (final volume 5 mL per well), and the percentage of cells expressing EGFP quantified 2 days post-transfection by flow cytometry. Only CHO-T cells cultivated in CD CHO could successfully be transfected, achieving transfection efficiencies of approximately 47% (Table 1). These results may be due to the presence of polyanionic molecules that can inhibit transfection due to the neutralization or destabilization of the positively charged polyplex.[12,17] Such molecules, including dextran sulfate, are routinely added to suspension media to prevent cell aggregation at high cell densities.

Table 1. Transfection efficiencies in various CD and SF growth media

Growth medium	Transfection efficiency (%)
CD CHO	47.7 ± 0.7
CDM4CHO	0
Excell CD CHO-3	0
Excell 302	0
PowerCHO 2	3.6 ± 0.05
ProCHO5	1.5 ± 0.01
SFM4CHO	0

(n = 2 ± SD).

PEI offers a significant cost advantage over other commercially available transfection reagents, making it an attractive option for scalable TGE with both academia and industry.[3,4] However, TGE processes utilizing more costly cationic-lipid formulations, such as Lipofectamine 2000, have also been shown to give significant protein yields with high transfection efficiencies and capacity to be transferred to large scale.[13,54] In addition, recent work performed by our group demonstrated significant improvements in recombinant protein titers using Lipofectamine 2000 in SF conditions.[42]

To determine whether PEI was the most suitable reagent for transfection in CD media, PEI was compared with Lipofectamine 2000. In CHO-S-SFMII, a protein-containing SF media formulation, Lipofectamine 2000 supported highly efficient transfection (greater than 65%), whereas transfection efficiencies with PEI were below 20% in all cases (Fig. 1(A)). However, with cultures in the fully defined growth media CD CHO, PEI-mediated transfection outperformed Lipofectamine 2000 based transfections (Fig. 1(B)). In a second study, PEI was compared with an alternative cationic-lipid formulation, Freestyle MAX, a transfection reagent for suspension cells in CD media. 12 mg L^{-1} of mAb was achieved using PEI compared with 9.5 mg L^{-1} with Freestyle Max (Fig. 1(C)).

Additional TGE studies (producing 15 mg L^{-1} of mAb after 14 days, data not shown) and data presented here demonstrates that PEI is suitable for transfections performed under CD conditions, however the recombinant mAb titers achieved remain low and require significant optimization.

Improved PEI-mediated transfection with the *Epi*-CHO system

Careful examination of the literature highlights that many variables contribute to the successful formation of PEI-plasmid DNA complexes.[12,19,29,55,56] In this work a multi-parametric study was performed to improve PEI-mediated transfections with the

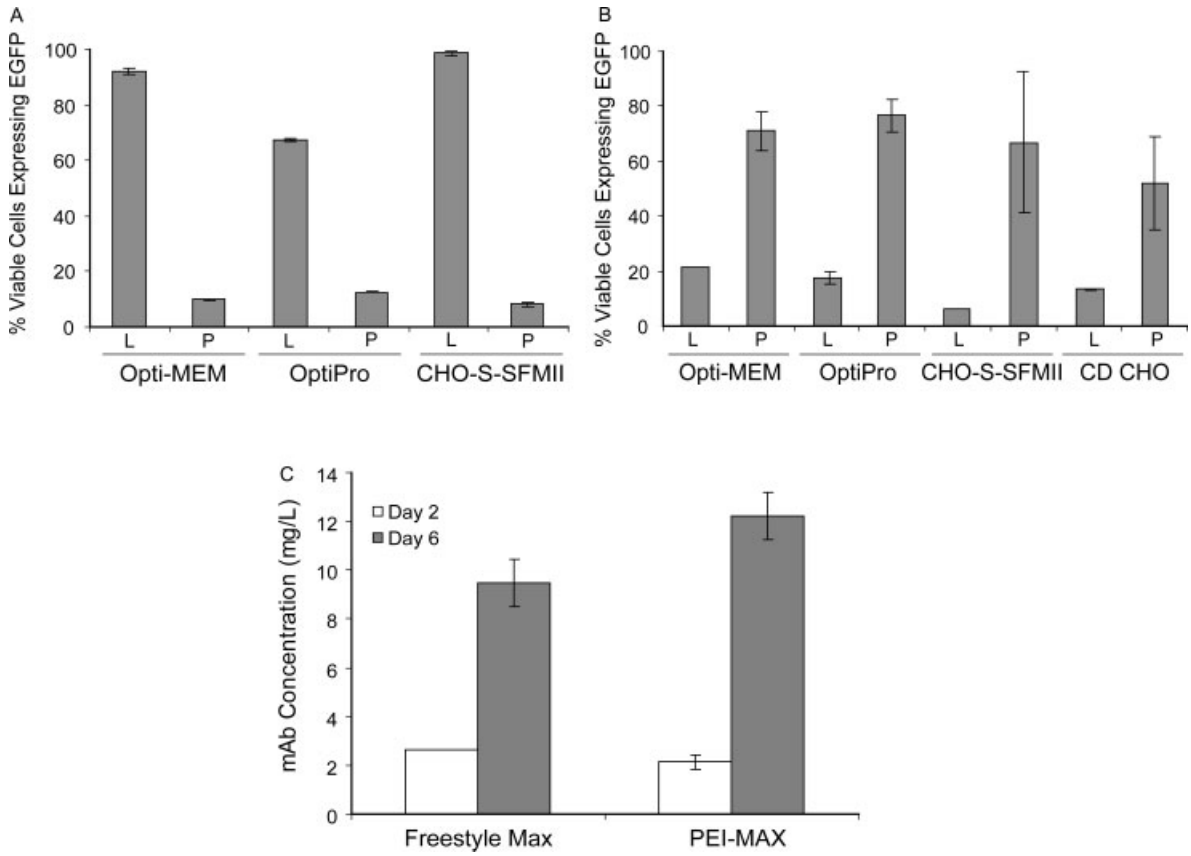

Figure 1. Comparison of reagents for transfection in CD media. CHO-T cells growing in CHO-S-SFMII (**A**) or CD CHO (**B**) were transfected with pEGFP-C1 complexed with either Lipofectamine 2000 ('L') or PEI ('P') in a final volume of 5 mL in six-well plates. Complexes were prepared in Opti-MEM, OptiPRO SFM, CHO-S-SFMII or CD CHO (CD CHO cultures only), with transfection efficiencies quantified 2 days post-transfection (n = 2 ± SD). (**C**) CHO-T cells growing in CD CHO were transfected with pPyEBV-Ab2HC and pPyEBV-Ab2LC (1 : 1) (w : w) complexed with either PEI or Freestyle MAX in a final volume of 5 mL in six-well plates. Samples for Ab2 quantification were collected at times indicated. n = 2 ± SD.

Epi-CHO expression system by examining cell seeding density, DNA : PEI ratios and DNA concentrations.

CHO-T cells growing in CD CHO were seeded at cell densities of 1.5×10^6, 2×10^6 and 3×10^6 cells mL^{-1} in six-well plates (final volume of 5 mL) and transfected with complexes composed of DNA : PEI ratios of 1 : 3, 1 : 4 and 1 : 5 (w : w), using 2 or 3 μg DNA mL^{-1} initial culture volume transfected. All complexes contained 5% of an EGFP expressing plasmid to monitor transfection efficiency, with mAb expression monitored over 14 days and compared with cultures transfected with our existing protocol.

EGFP mean fluorescence intensities (FIs) were similar for all transfectants irrespective of DNA concentration (Fig. 2(A) and 2(B)). Interestingly, transfection efficiencies appeared not to be dependent on any one variable tested, with no consistent trend detected. Cultures transfected with 2 μg DNA mL^{-1} culture generated the highest mAb concentrations with seeding densities of 3×10^6 cells mL^{-1} for DNA : PEI ratios of 1 : 3, 1 : 4 and 1 : 5 (46, 42 and 43 mg L^{-1} of mAb, respectively) (Fig. 2(C)), with these conditions also generating the highest viable cell densities (Fig. 2(E)). Transfection conditions incorporating lower ratios of DNA : PEI and lower seeding densities appeared to generate lower mAb titers (<40 mg L^{-1}) and in turn lower viable cell densities, yet they sustained higher cell viabilities post day 9 (Fig. 2(E)). Furthermore, the use of higher DNA concentrations appeared to have minimal effect on viable cell densities or cell viability compared to those transfected with lower DNA concentrations (Fig. 2(F)) and did not provide any significant improvement in mAb titers (Fig. 2(D)).

To confirm these conditions, an additional study in 125 mL shake flasks was performed (final volume of 20 mL) comparing three of the best conditions identified in the above study. The experiment verified that transfecting cells at densities of 3×10^6 cells mL^{-1} with a DNA : PEI ratio of 1 : 4 consistently generated higher EGFP expression (Fig. 3(A)), higher mAb titers (40–45 mg L^{-1}) (Fig. 3(B)), and favorable cell growth and viability (Fig. 3(C)). By examining all variables in a single study, we have identified an improved PEI-based transfection process using CD media which routinely generates 45 mg L^{-1} of mAb under batch conditions. This represents a 3-fold improvement over our initial starting conditions.

Delayed transfer to mild hypothermic conditions improves mAb production

Mild hypothermic conditions (27–33 °C) have previously been employed in recombinant protein expression studies to improve yields with both stable and transient expression systems.[29,31,36] Culturing cells under these conditions provides enhanced mRNA stability and elevated culture viabilities through slower cell metabolism and a reduced rate of toxic metabolite accumulation,[31,33,34,57] without any detrimental effect on post-translational modifications such as glycosylation.[29,31,55,58] With stably transfected cell lines, several reports have highlighted

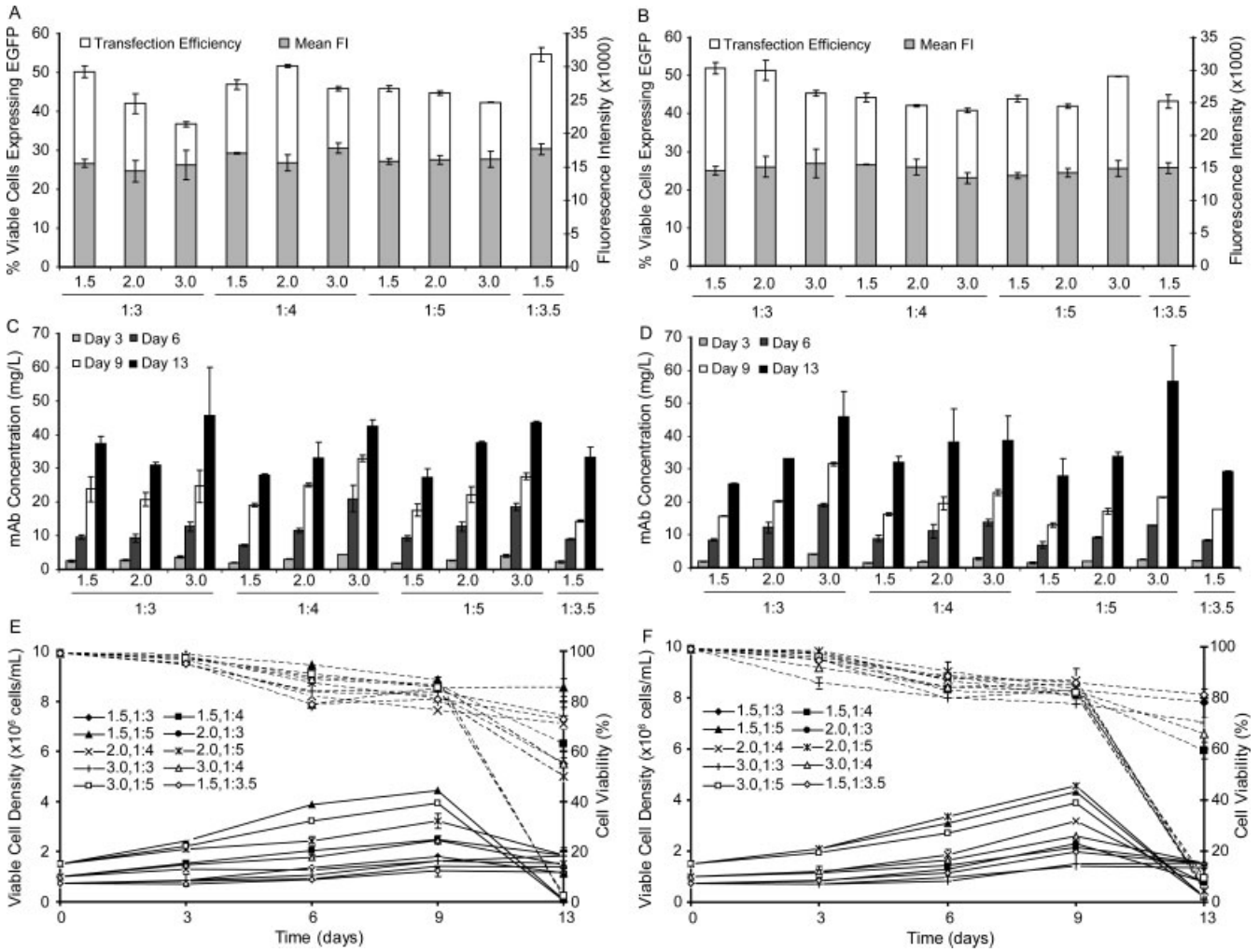

Figure 2. The effects of cell density, DNA concentration and DNA : PEI ratio on transfection efficiency. CHO-T cells growing in CD CHO were transfected to express Ab2 and EGFP in a final volume of 5 mL in six-well plates. Cultures were seeded at cell densities of 1.5×10^6, 2×10^6 and 3×10^6 cells mL^{-1} and transfected with DNA : PEI ratios of 1 : 3, 1 : 3.5, 1 : 4 and 1 : 5 (w : w). At 4 h post-transfection, cultures were diluted 1 : 1 (v : v) with ProCHO5 supplemented with 8 mmol L^{-1} GlutaMAX, 0.4% (v/v) ACA and 100 μg L^{-1} IGF-1 and incubated at 32 °C. Transfection efficiencies and mean FIs at day 3 post-transfection are displayed for samples transfected with 2 μg (**C**) or 3 μg (**D**) DNA mL^{-1} of culture. Ab2 concentrations at times indicated are displayed for cultures transfected with 2 μg (**A**) or 3 μg (**B**) DNA mL^{-1} of culture. Viable cell density and cell viability were determined at the time points indicated for cells transfected with 2 μg (**E**) or 3 μg (**F**) DNA mL^{-1} of culture. n = 2 ± SD.

substantial improvements in titer by initially culturing cells at 37 °C until mid-to-late logarithmic phase and then transferring to conditions of mild hypothermia.[30,59,60] However, with TGE, shifts immediately post-transfection have been shown to give the greatest improvements in titer.[31] This is presumably due to a reduction in the rate of plasmid loss when cells are cultured at a lower temperature and undergoing slower cell division. Using the *Epi*-CHO system we speculated whether it was possible to use an alternative approach, in which cells are maintained at 37 °C post-transfection to promote cell growth and DNA replication, before a transfer to mild hypothermia to boost protein expression. To investigate this, transfected CHO-T cultures expressing Ab2 and EGFP, were transferred to 32 °C at 4 (the existing time of transfer used with *Epi*-CHO), 48 and 96 h post-transfection and diluted with fresh media at the time of transfer (1 : 1, v : v) and/or immediately post-transfection (1 : 2, v : v) ('double or single feed') to promote extended culture longevity and viability. In these experiments we utilized ProCHO5 for the dilutions post-transfection as this media maintains high cell densities over extended culture periods.[42]

Cultures transferred to 32 °C at 4 h had higher transfection efficiencies and mean FIs compared with those maintained at 37 °C until days 2 or 4 (Fig. 4(A)), which may be attributed to the faster dilution rate of pEGFP-C1 plasmid DNA (replication deficient) at 37 °C. Transfectants transferred to 32 °C at 48 h post-transfection generated 64 mg L^{-1} of mAb after 16 days with a single feed compared to those transferred 4 h post-transfection (~50–55 mg L^{-1}) (Fig. 4(B)), with only a slight reduction in average specific productivity (1.3 vs 1.6 pg per cell per day) (Fig. 4(C)). More importantly, using the same initial transfection volume, those transferred to 32 °C at 48 h post-transfection with a double feed maintained comparable average specific productivities (1.4 pg per cell per day) and ~2.5-fold increase in total mAb produced compared to our previous protocol (~1.4 mg). Cultures transferred at 96 h post-transfection also generated improved mAb quantities with a double feed (~2.7 mg), yet this was not mirrored with the single feed variant. Typically, cultures receiving a double feed had lower viable cell densities but sustained higher cell viabilities and levels of mAb production (compared with their single feed

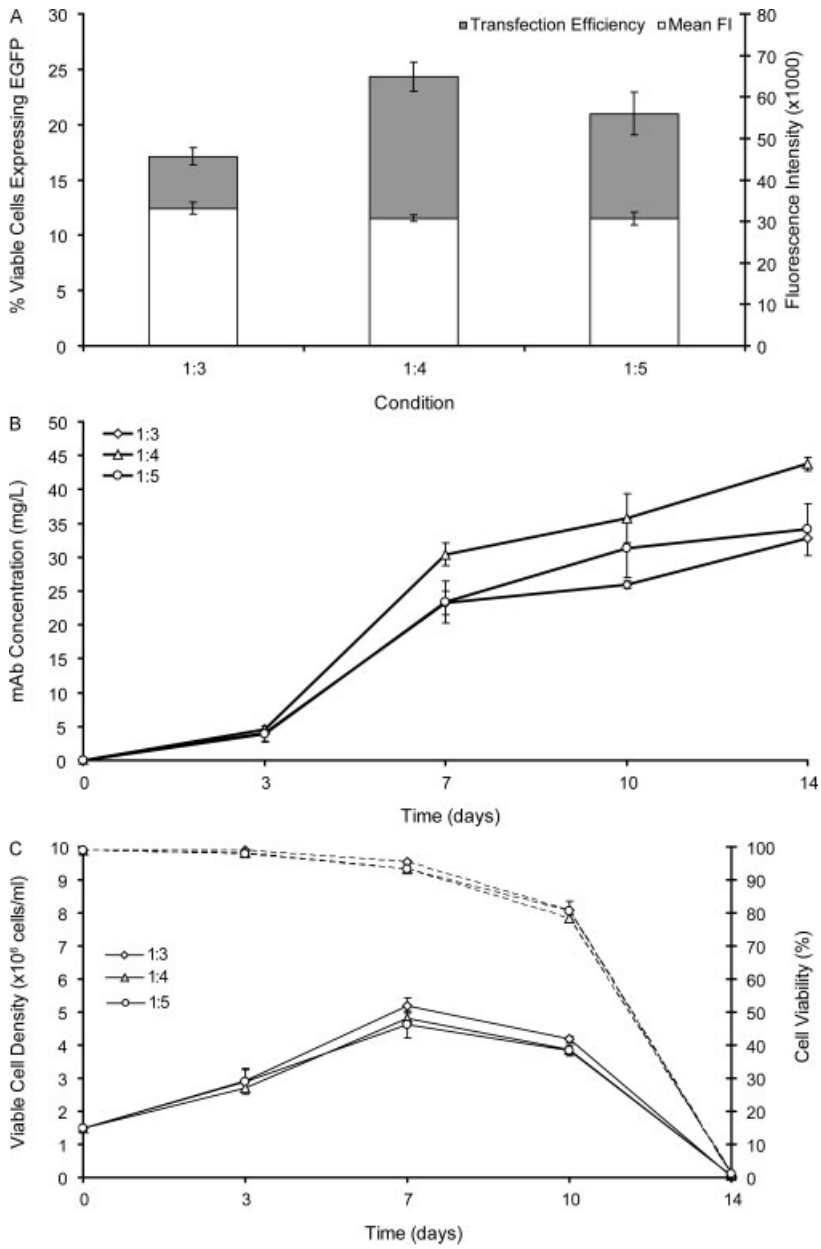

Figure 3. Improved PEI-mediated transfection with the *Epi*-CHO system. CHO-T cells (seeded at 3×10^6 cells mL^{-1}) growing in CD CHO were transfected with pPyEBV-Ab2HC : pPyEBV-Ab2LC : pEGFP-C1 (48.75 : 48 : 75 : 2.5, w : w) in a final volume of 20 mL in 125 mL shake flasks. Cultures were transfected with 2 μg DNA/mL of culture at DNA : PEI ratios of 1 : 3, 1 : 4 and 1 : 5 (w : w). At 4 h post-transfection, cultures were diluted 1 : 1 (v : v) with ProCHO5 supplemented with 8 mmol L^{-1} GlutaMAX, 0.4% (v/v) ACA and 100 μg L^{-1} IGF-1 and incubated at 32 °C. (**A**) Transfection efficiencies and their mean FIs at day 2. (**B**) Ab2 concentrations, (**C**) viable cell density and cell viability were determined at time points indicated. n = 3 ± SD.

controls) (Fig. 4(D)). Fed-batch strategies have been employed with HEK.293E cells (plasmid replication and retention competent), whereby feeding post-transfection delivered improved titers of recombinant protein.[61–63] Previous reports with CHO-cell based TGE have required continued media exchange throughout the expression study in order to enhance final yields.[29,41] In this study, the capacity for culture volume to be expanded six times the initial transfection volume with the *Epi*-CHO system to increase biomass and recombinant protein titer, without any increase in the requirement for DNA or transfection reagent (nor media replacement), represents the first report for such a strategy to be applied with CHO cell-based TGE. Additional studies performed verified a recent report,[31] that higher mAb titers and prolonged cell viability are witnessed in cultures cultivated at mild hypothermia compared with those maintained at 37 °C, with slower rates of glucose consumption and lower rates of toxic metabolite accumulation also observed (data not shown).

The advantages of a delayed transfer to mild hypothermia and culture expansion post-transfection with the *Epi*-CHO system

We have previously demonstrated that CHO-T transfectants generated improved human growth hormone, EGFP and mAb quantities compared with CHO-K1 transfectants maintained at 37 °C or transferred to 32 °C immediately post-transfection.[41,42] Using our new protocol incorporating a delayed transfer to mild hypothermia and additional feeding, we compared the *Epi*-CHO system with

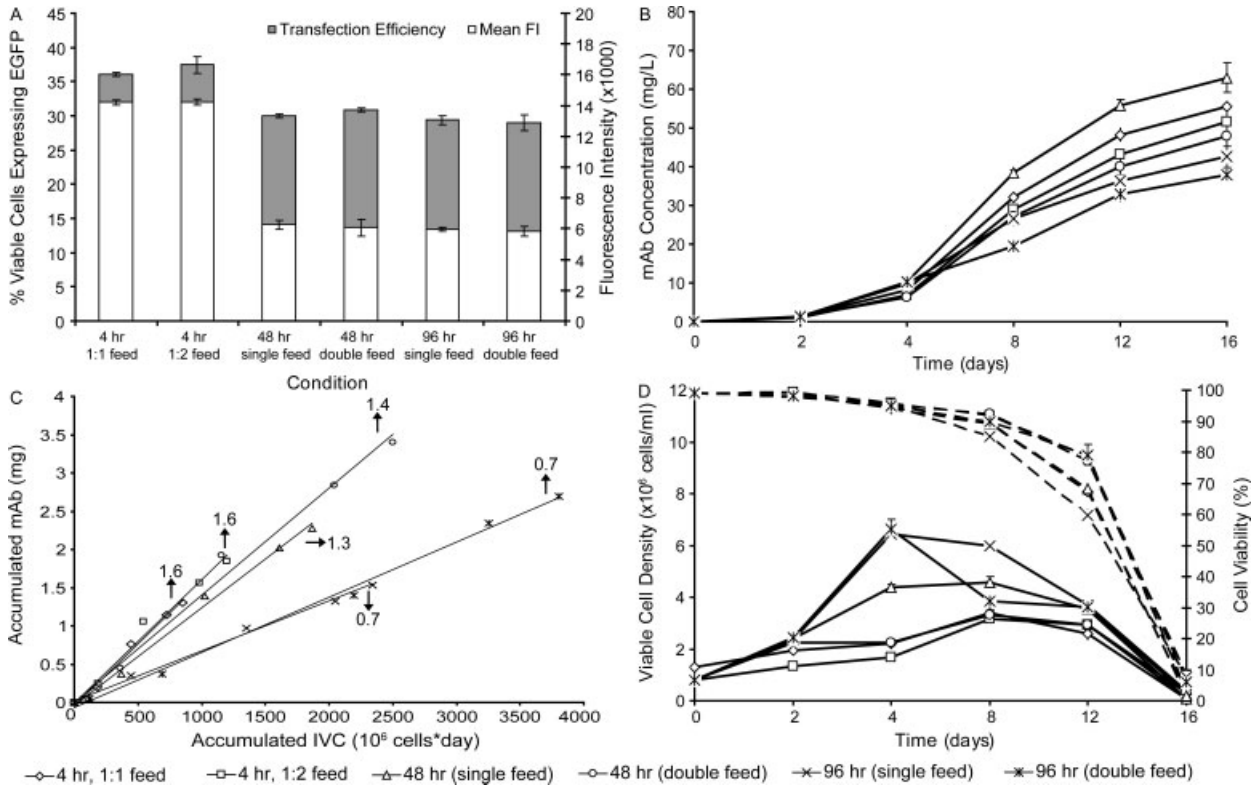

Figure 4. Delayed transfer to mild hypothermia improves recombinant mAb titers. CHO-T cells growing in CD CHO were transfected to express Ab2 and EGFP (pPyEBV-Ab2HC : pPyEBV-Ab2LC : pEGFP-C1) (48.75 : 48.75 : 2.5, w : w) using the new, optimized conditions in an initial volume of 12.5 mL in 125 mL shake flasks at 37 °C. All cultures were diluted post-transfection with supplemented ProCHO5. Cultures transferred 4 h post-transfection were fed 1 : 1 or 1 : 2 (v : v). Cultures transferred to 32 °C at 48 and 96 h post-transfection were fed 1 : 2 (v : v) 4 h post-transfection ('single feed') or additionally at the time of transfer (1 : 1, v : v)) (double feed). (**A**) Transfection efficiencies and mean FIs at day 2. (**B**) Ab2 concentrations were determined at time points indicated. (**C**) Accumulated Ab2 (mg) plotted against the accumulated IVC number over the 16 day expression study. Average specific productivities (pg per cell day^{-1}) over the study are listed (↑) (**D**) Viable cell densities and cell viability were determined at time points indicated. n = 3 ± SD.

transfectants deficient for plasmid replication and/or retention. *Epi*-CHO generated higher mAb concentrations (Fig. 5(A)) with lower viable cell densities (Fig. 5(C)), in turn delivering larger quantities of mAb and average specific productivities (~3.4 mg and 1.1 pg per cell day^{-1}) compared with replication and/or retention deficient transfectants (<1.6 mg and <0.3 pg per cell day^{-1}) (Fig. 5(B)). The generation of 155 μg of mAb μg^{-1} of plasmid DNA with the *Epi*-CHO system compared with 70 μg of mAb μg^{-1} DNA using the best-case non-episomal control clearly illustrates the advantages of the *Epi*-CHO system and its capacity to be expanded to larger culture volumes post-transfection, without any increase in the requirement for DNA or transfection reagent.

Screening of media and feeds to enhance TGE

In an attempt to further enhance TGE with *Epi*-CHO, we examined several CD media and supplements for their capacity to sustain higher viable cell densities at mild hypothermia post-transfection and in turn drive further improvements in mAb production. *Epi*-CHO transfectants expressing Ab2 were fed with various commercial media at 4 and 48 h post-transfection including ProCHO5 (as described), ProCHO5 supplemented with Power Feed A (Lonza) (20% v/v), CD CHO and CD CHO supplemented with Efficient Feed's A and B (Invitrogen) (7.5% each, v/v). With all aspects considered, the greatest improvements in TGE were seen with transfectants fed with CD CHO, supplemented with Efficient Feeds A and B. This medium generated improved viable cell densities over a longer period (Fig. 6(D)), which attributed to improved recombinant mAb titers of 80 mg L^{-1} (compared to 48 mg L^{-1} with ProCHO5) (Fig. 6(B)) and an average specific productivity of 2.4 pg per cell day^{-1} (~1.6 pg per cell day^{-1} with ProCHO5) (Fig. 6(C)). This feeding strategy further enhanced the efficiency of the *Epi*-CHO system, delivering 279 μg of mAb μg^{-1} DNA transfected compared with 167 μg of mAb μg^{-1} DNA when using ProCHO5. Transfection efficiencies and EGFP mean FIs were comparable with all transfectants (Fig. 6(A)). Average cell diameters were comparable with all media except with the slower growing CD CHO fed culture, where they were significantly larger (Fig. 6(E)). In addition, the slower growth in this medium provided a higher average specific productivity of 3.6 pg per cell day^{-1} and a final concentration of 69 mg L^{-1}. The improvements in production and growth seen with cultures supplemented with Efficient Feeds were facilitated by more efficient use of higher glucose concentrations (Fig. 6(G)) and slower rates of lactate and ammonium production (Fig. 6(H) and 6(I)), promoting an elevated and better-sustained culture pH compared with ProCHO5 (Fig. 6(F)).

Co-expression of XBP-1 facilitates higher concentrations of mAb at mild hypothermia

XBP-1 has been suggested to play a key role in regulating the secretion pathway of mammalian cells.[51] In stable expressing cell lines, over-expression of XBP-1 has been used to enhance secretory

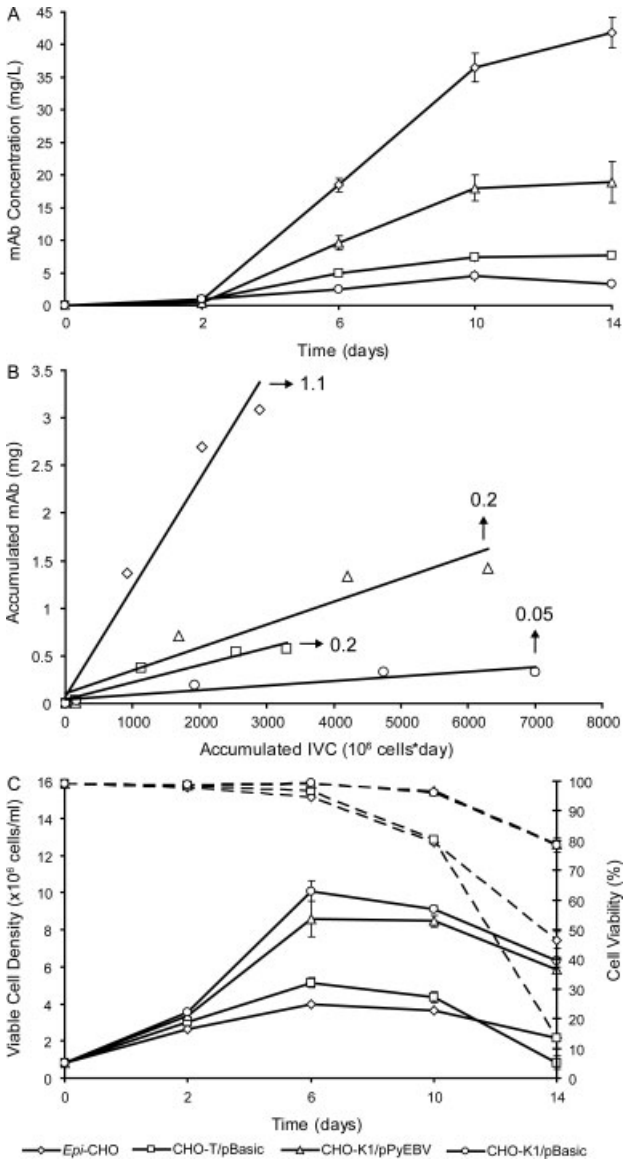

Figure 5. Delayed transfer to mild hypothermia provides the greatest benefits to *Epi*-CHO. CHO-T and CHO-K1 cells growing in CD CHO were transfected (optimized PEI conditions) with pPyEBV or pBasic vectors to express Ab2 in an initial volume of 12.5 mL in shake flasks at 37 °C. 4 h post-transfection, cultures were diluted 1 : 2 (v : v) and maintained at 37 °C. 48 h post-transfection, cultures were diluted 1 : 1 (v : v) and transferred to 32 °C. (**A**) Ab2 concentrations were determined at time points indicated. (**B**) Accumulated Ab2 (mg) plotted against the accumulated IVC number over the 14 day expression study. Average specific productivities (pg per cell day^{-1}) over the 16 day expression study are listed (↑) (**C**) Viable cell density and cell viability were determined at the time points indicated. n = 3 ± SD.

capacity, yet improvements in protein production are witnessed once concentrations exceed the secretion capacity of the host cell.[50,64] Ku and colleagues witnessed secretion saturation in CHO-K1 cells transfected with increasing dosages of plasmid DNA expressing erythropoietin and that through the co-expression of XBP-1, alleviated this bottleneck and generated improvements in final recombinant protein yield.[64] With respect to TGE, the incorporation of efficient gene delivery methods will contribute to higher levels of expression and in turn improved recombinant protein titers as shown in this report. To determine whether such methods imposed secretion stress on transfected cells we examined whether the co-expression of the active spliced variant of murine XBP-1 could improve TGE with the *Epi*-CHO system.

Transfections with CHO-T cells were performed in six-well plates, with cultures either maintained at 37 °C or transferred to 32 °C 4 h post transfection (cultures were diluted 1 : 2 (v : v) at this time with growth media with no additional feeding during the study). Co-transfecting cells with 2.5% pcDNA3.1-XBP1 had no effect on product titer in cultures maintained at 37 °C (26 mg L^{-1} versus 23 mg L^{-1} for the control) (Fig. 7(A)). However, cultures transferred to 32 °C delivered a 37% increase in mAb titer, generating 52 mg L^{-1} of mAb after 10 days compared with control transfections (38 mg L^{-1}). Transfecting with a higher percentage of XBP-1 plasmid (5%) did not provide any further improvements, and the highest titers were again achieved with the cultures transferred to mild hypothermia (43 mg L^{-1}). Viable cell densities and cell viability were comparable between all transfectants and their respective incubation temperature (Fig. 7(B))

Finally, transfections performed in 125 mL shake flasks (final volume 25 mL) verified the above results, with those transferred to mild hypothermia and co-transfected with 2.5% pcDNA3.1-XBP1 generating 51 mg L^{-1} compared with 40 mg L^{-1} produced by control transfectants (Fig. 7(C)). Expressing XBP-1 with the *Epi*-CHO system when using our previous un-optimized PEI-mediated transfection protocol delivered no improvement in mAb titer (data not shown). The results presented here indicate that the co-expression of XBP-1 with TGE systems incorporating an efficient transfection strategy and mild hypothermia may alleviate secretion bottlenecks that arise from improved mRNA stability and increased transcript levels. Ku and colleagues demonstrated that XBP-1 had a positive effect on transient EPO production with culture maintained at 37 °C for 2 days.[64] The lack of improvements in mAb production seen in this study at 37 °C suggests that secretion is not saturated under these conditions over a period of 10 days. This may be due to elevated cell growth and a faster rate of plasmid dilution to daughter cells after consecutive rounds of cell division, which in turn reduces transcript levels and reduces the burden on secretion machinery. Alternatively, considering cells were transfected with a small amount (2.5% (w/w)) of an XBP-1 expressing plasmid that was both replication and retention deficient, dilution of XBP-1 expression at an earlier stage of the process may also explain why no improvements in titer were observed. While such approaches to improving TGE remain in their infancy, these experiments further demonstrate the potential of co-transfecting cells with transcription or growth factors to enhance recombinant protein yields from TGE.[65]

CONCLUSION

Through the examination of variables and parameters critical for efficient transfection, we have developed an enhanced TGE process in CD conditions for the *Epi*-CHO system. Using a modified PEI as the transfection reagent, we optimized parameters including cell density, PEI : plasmid DNA ratio and complex formation media that contribute to the efficient uptake of plasmid DNA into CHO cells. Through the implementation of these parameters, identifying the ideal time for the transfer of transfected CHO cells to mild hypothermic conditions post-transfection and the screening of various CD media and supplements, we achieved a five-fold improvement in mAb concentration (80 mg L^{-1}) over our initial process and a two-fold improvement in total mAb produced compared with cultures not fed or transferred to 32 °C at 4 h

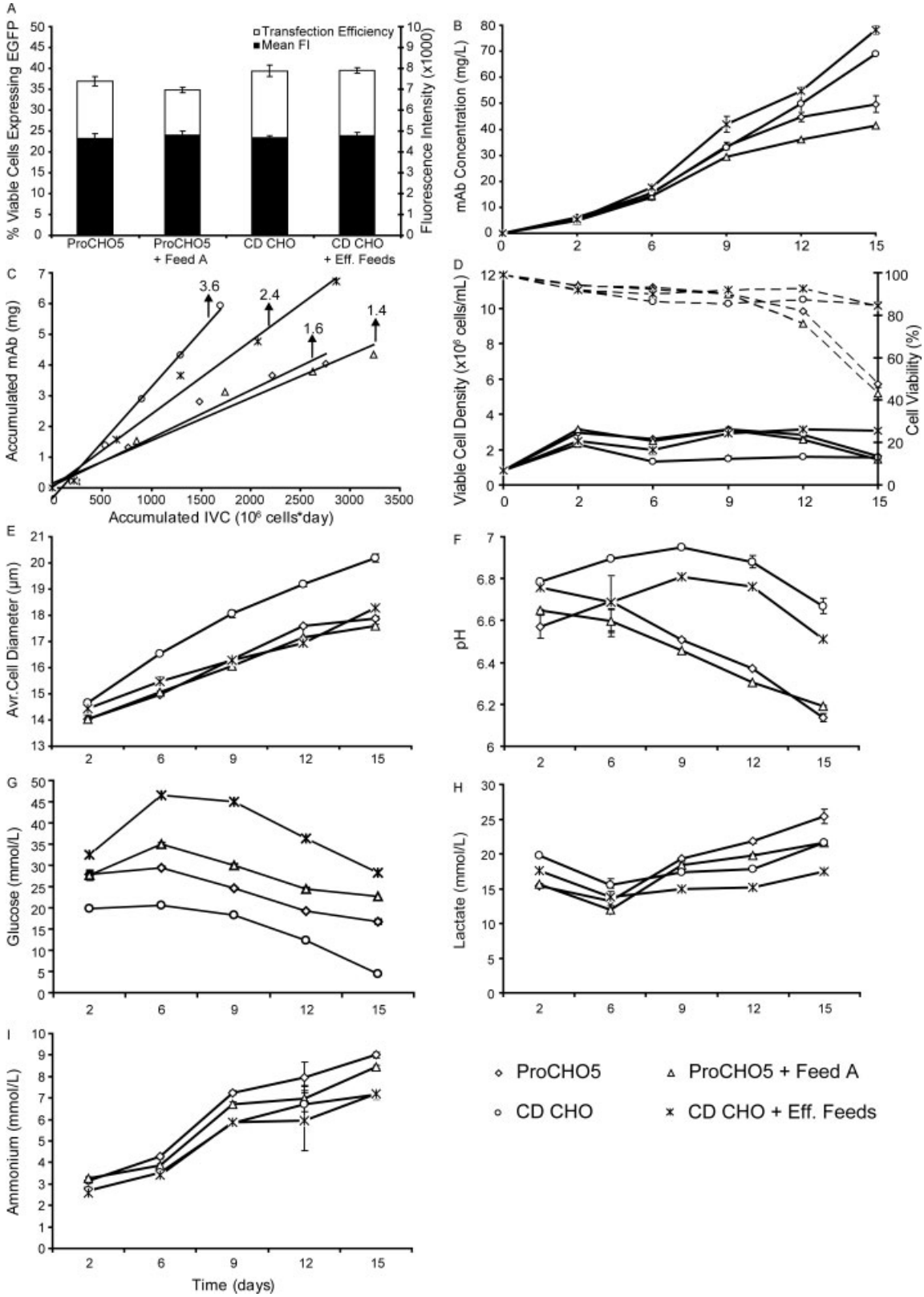

Figure 6. Screening of CD media and supplements to improve TGE with *Epi*-CHO. Transfected CHO-T cells expressing Ab2 and EGFP (pPyEBV-Ab2HC : pPyEBV-Ab2LC : pEGFP-C1) (48.75 : 48.75 : 2.5, w : w) were fed with either ProCHO5, ProCHO5 supplemented with Power Feed A, CD CHO or CD CHO supplemented with Efficient Feeds A and B. All feeds were administered at 4 h (1 : 2, v : v) and 48 h (1 : 1, v : v) post-transfection. (**A**) Transfection efficiencies and mean FIs were quantified at day 2. (**B**) Ab2 concentrations in supernatants at the time points indicated. (**C**) Accumulated Ab2 (mg) plotted against the accumulated IVC number over the 15 day expression study. Average specific productivities (pg per cell day^{-1}) over the study are listed (↑). (**D**) Viable cell density, cell viability (**E**) average cell diameter, (**F**) pH, (**G**) glucose concentration, (**H**) lactate concentration and (**I**) ammonium concentration were determined at the time points indicated. n = 3 ± SD.

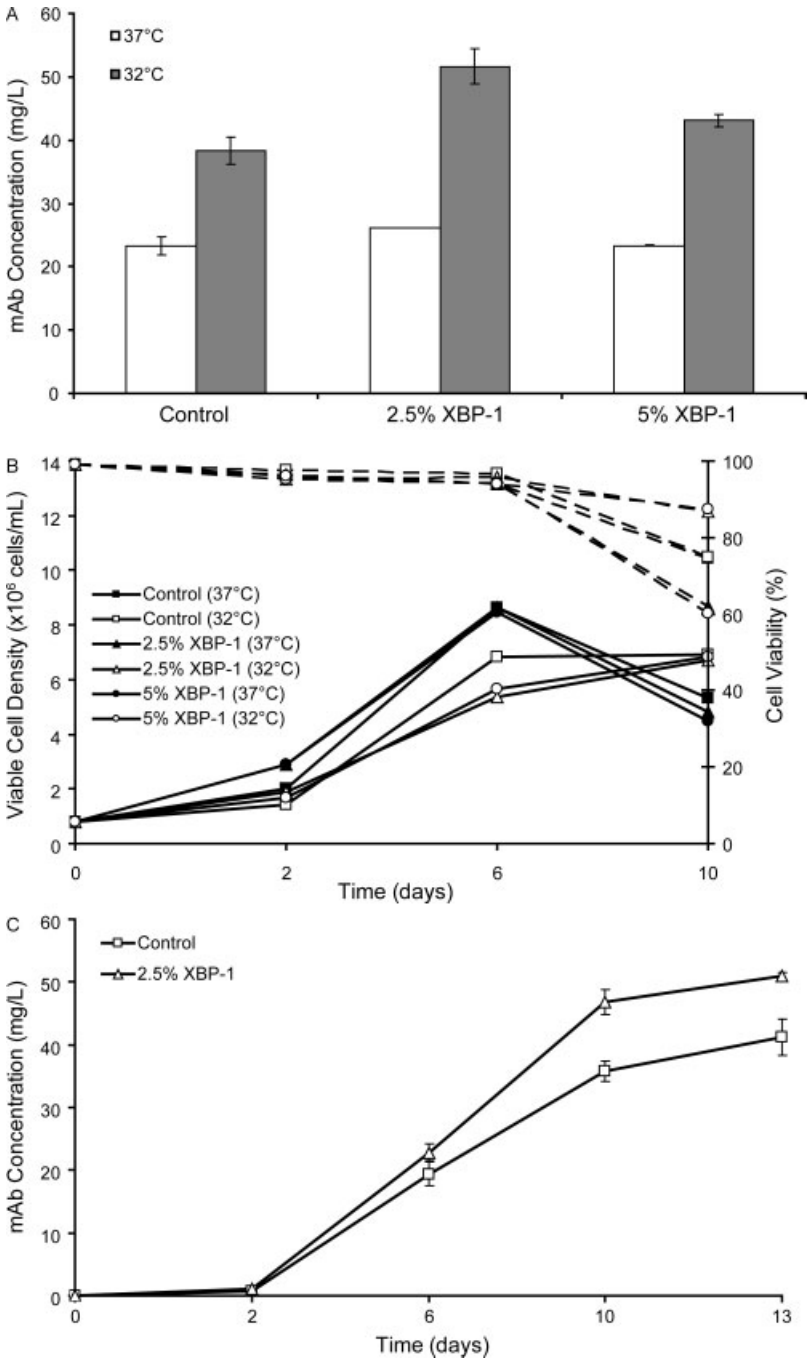

Figure 7. Co-expression of XBP-1 leads to higher titers of mAb at mild hypothermia. CHO-T cells growing in CD CHO were transfected with equal amounts of pPyEBV-Ab2HC and pPyEBV-Ab2LC and either 2.5% (w : w) pEGFP-C1 ('control') or pcDNA3.1-XBP1 in an initial volume of 4 mL in six-well plates at 37 °C. An additional series of transfectants with 5% pcDNA3.1-XBP1 were also performed. Cultures were transfected with 2 μg DNA mL^{-1} of culture at a ratio of 1 : 4 (w : w) with PEI. All cultures were diluted (1 : 2, v : v) 4 h post-transfection with CD CHO supplemented to a final concentration of 7.5% (v : v) Efficient Feeds A and B (each). Finally, transfectants in each case were either maintained at 37 °C or transferred to 32 °C at 4 h. (**A**) Ab2 concentrations at day 10, (**B**) viable cell densities and cell viabilities at the time points indicated are displayed (n = 2 ± SD). (**C**) CHO-T cells were transfected with pPyEBV-Ab2HC : pPyEBV-Ab2LC : pEGFP-C1 ('control') or pcDNA3.1-XBP1 (48.75 : 48 : 75 : 2.5, w : w) in a final volume of 25 mL in 125 mL shake flasks. Transfectants were transferred to 32 °C at 4 h post-transfection and Ab2 concentrations quantified at the time points indicated. n = 3 ± SD.

post-transfection (including replication-deficient transfectants). Using this strategy we were also able generate 279 μg of mAb μg^{-1} of plasmid DNA with the *Epi*-CHO system, representing 4X increase over a non-episomal system. Finally, through the co-expression of XBP-1 with the *Epi*-CHO system, improvements in mAb titers can also be achieved at mild hypothermic conditions when incorporating an efficient gene delivery protocol.

The approaches we have defined with systems like *Epi*-CHO provide an alternative to overcoming the issue of low biomass accumulation that is often associated with CHO-cell based transient processes incorporating mild hypothermia. Critically,

these results highlight the scalability and efficiency of the *Epi*-CHO system, whereby the initial transfection volume can be increased six-fold without any required increase in DNA or transfection reagent, to deliver superior recombinant protein yields over replication-deficient CHO-K1 transfectants. In summary we have developed a highly efficient and cost-effective TGE system in CHO cells using defined media which is suitable for the rapid production of significant quantities of a drug candidate in the pre-clinical assessment stage.

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